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Title: Visualization of the synergistic effect of lithium acetate and single-stranded carrier DNA on *Saccharomyces cerevisiae* transformation

Short title: Visualization of the synergistic effect

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Abstract

Transformation is an indispensable method for the genetic manipulation of cells. *Saccharomyces cerevisiae* can be transformed by incubating intact cells and plasmid DNA in the presence of polyethylene glycol alone. Lithium acetate and single-stranded carrier DNA enhance the transformation efficiency, but the mechanism underlying this enhancement has remained elusive. In this study, we first confirmed that lithium acetate and single-stranded carrier DNA synergistically improve the transformation efficiency of *S. cerevisiae* intact cells. We then used transmission electron microscopy to observe the cell walls of yeast incubated with both lithium acetate and single-stranded carrier DNA in the presence of negatively charged Nanogold (in this context, a mimic of DNA). Under these conditions, the cell walls exhibited protruded, loose, and porous structures. The Nanogold was observed within the cell wall, rather than on the surface. We also made observations using YOYO-1, a fluorescent DNA probe. Based on the transmission electron microscopy and fluorescence data, we speculated that single-stranded carrier DNA covers the whole cell and enters, at least partially, into the cell wall structure, causing the cell wall to become protruded, loose, and porous; meanwhile, LiAc gives effect on the cell wall. Together, the two compounds synergistically enhance transformation efficiency and frequency.

Key words: *Saccharomyces cerevisiae*, transformation, lithium acetate, single-stranded carrier DNA, transmission electron microscopy, Nanogold.

1 Introduction

Transformation, in which exogenous plasmid DNA is introduced into cells, is an indispensable technique for genetic modification. In *Saccharomyces cerevisiae*, the spheroplast transformation method was established in 1978 (Hinnen, et al. 1978). Later, the lithium method for transforming intact *S. cerevisiae* cells was developed (Ito, et al. 1983). In the lithium method, monovalent cations (generally lithium, but also rubidium), enhance the transformation efficiency (the number of transformants per μg plasmid DNA) of intact *S. cerevisiae* cells. Ito et al. described the effect of transformation reagents in detail, demonstrating (i) incubation of intact cells with polyethylene glycol (PEG) and plasmid DNA is essential for transformation; (ii) short-term incubation of intact cells with PEG and plasmid DNA at 42°C (heat shock) enhances the transformation efficiency; (iii) transformation of the cells is most effective at the mid-log phase; and (iv) lithium acetate (LiAc) is the most effective monovalent cation (Ito, et al. 1983). Subsequently, Gietz et al. modified the original lithium method and succeeded in improving its efficiency by immediately mixing washed intact cells with PEG, LiAc, plasmid DNA, and single-stranded carrier DNA (ssDNA), and incubating them at 42°C for 40–60 min (Gietz, et al. 1992, Gietz, et al. 1995, Gietz and Woods 2002, Schiestl and Gietz 1989). The modified lithium method has been referred to as the LiAc/ssDNA/PEG method (Gietz and Woods 2002). Intact cells can be transformed without the addition of LiAc by incubating the cells with PEG and plasmid DNA at 30°C and then heat-shocking at 42°C (heat shock) (Hayama, et al. 2002, Yamakawa, et al. 1985). From these results, we can conclude that PEG is required for transformation, while LiAc and ssDNA both serve to enhance transformation efficiency. The

mechanism underlying this enhancement has remained elusive.

LiAc increases the permeability of intact cells to nucleic acid (Brzobohaty and Kovac 1986) and YOYO-1 (Zheng, et al. 2005) but has no effect on the transformation frequency (the transformation efficiency per viable cell) of spheroplasts (Chen, et al. 2008). Furthermore, Chen et al. observed by scanning electron microscopy that the surfaces of intact cells incubated with LiAc at 30°C for 30 min became much rougher and more wrinkled, whereas the surfaces of intact cells incubated without LiAc as well as those of spheroplasts remained homogenous and smooth (Chen, et al. 2008). Thus, it has been proposed that LiAc helps plasmid DNA to pass through the cell wall, but not the plasma membrane (Chen, et al. 2008, Kawai, et al. 2010). However, it should be stressed that the aforementioned studies examined the role of LiAc alone, in the absence of ssDNA. Previous studies reported that ssDNA had no effect on the transformation efficiency of spheroplasts (Schiestl and Gietz 1989), leaving open the possibility that ssDNA could have an effect on the cell wall in the presence of LiAc.

In this study, we focused initially on the effect of ssDNA alone on transformation efficiency; our results revealed that ssDNA enhanced transformation, albeit less efficiently than LiAc alone. Subsequently, we shifted focus to the synergistic effect of LiAc and ssDNA together. Furthermore, we visualized the synergistic effects of LiAc and ssDNA on the cell wall using transmission electron microscopy (TEM).

Materials and methods

Strains

S. cerevisiae BY4742 (*MAT α leu2 Δ 0 lys2 Δ 0 ura3 Δ 0 his3 Δ 1*) was purchased from EUROSCARF and cultivated under standard yeast growth conditions (Sherman 2002).

Transformation

Transformation of intact *S. cerevisiae* cells was performed as described (Gietz and Woods 2002), with slight modifications. In brief, for each transformation reaction, *S. cerevisiae* cells were collected from 5.5 ml yeast–peptone–dextrose (YPD) culture (Sherman 2002) during log phase (approximate A_{600} of 0.5). The cells were washed once with sterilized distilled water (SDW) and incubated at 42°C for 20 min in 42 μ l suspension consisting of 2.0 μ l plasmid DNA [pRS415 (Sikorski and Hieter 1989); 0.10 μ g/ μ l], 30 μ l 50% (w/v) PEG (P3640; Sigma, St. Louis, MO), 4.5 μ l 1.0 M LiAc, and 6.0 μ l ssDNA (2.0 mg/ml; boiled and cooled salmon testis DNA, D1626, Sigma). Final concentrations of reagents in the suspension were 4.76 μ g/ml pRS415, 36% PEG, 10.7 mM LiAc, and 0.29 mg/ml ssDNA. To examine the individual contributions from LiAc and ssDNA, LiAc (1.0 M, 4.5 μ l) was replaced with rubidium acetate (RbAc) (1.0 M, 4.5 μ l) or SDW (4.5 μ l), and ssDNA (6.0 μ l) was replaced with SDW (6.0 μ l). After incubation, cells were resuspended in 1.0 ml SDW. After 10 μ l of the suspension was removed and diluted in SDW, the cells in the remaining suspension were collected and spread onto selective solid synthetic complete (SC) medium without leucine (Sherman

2002). Viable cells were counted by spreading the diluted suspension on YPD solid medium.

In this article, transformation efficiency is defined as the number of transformants per μg plasmid DNA (pRS415), while transformation frequency is defined as the transformation efficiency divided by the number of viable cells.

Transmission electron microscopy (TEM)

Cells were subjected to the transformation procedure (Gietz and Woods 2002) as above, but were incubated in 44 μl suspension containing 4.0 μl of negatively charged Nanogold (100 pmol/ μl in ultra pure water, 1.4 nm in diameter; Nanoprobes, Yaphank, NY) instead of 2.0 μl pRS415. Cells were collected by centrifugation, and the supernatant was removed completely. Next, cells were resuspended in 2% paraformaldehyde and 2% glutaraldehyde, fixed overnight, frozen in liquid propane using copper disks (Maxtaform Grid II HF51, Nisshin EM), substituted in 2% osmium tetroxide in acetone plus 3% water, dehydrated, and embedded in Quetol-651 (Nisshin EM) (Baba 2008). Ultra-thin sections (approximately 80 nm thick) were cut with a diamond knife on an LKB2088 ultramicrotome V (LKB-Produkter AB, Stockholm, Sweden), treated with GoldEnhance (Nanoprobes) for 1 min at room temperature, and stained with 2% aqueous uranyl acetate followed by lead staining (Sigma). The sections were examined using a JEM-1200EX microscope (JEOL, Tokyo, Japan) at 80 kV.

Fluorescence microscopic observation

Fluorescence microscopy was performed using a BX51 system (Olympus, Tokyo, Japan) equipped with a CoolSNAP camera (Quantitative Imaging Corporation, Burnaby, Canada) and filter (U-MNIBA2). YEp13 (Broach, et al. 1979) was labelled with YOYO-1 as described (Pham, et al. 2011). Transformation of *S. cerevisiae* cells was conducted by incubating 42 μ l of cell suspension at 42°C for 20 min as above, but substituting 2.0 μ l 1.0 μ M YOYO-1 alone or 2.0 μ l YOYO-1-labelled YEp13 (90 μ g [12.7 pmol] in 1.0 μ M YOYO-1) for 2.0 μ l pRS415.

Results and discussion

Synergistic effect of LiAc and ssDNA

To understand the individual contributions of LiAc and ssDNA, and of both compounds in combination, we compared the transformation efficiency and frequency obtained by incubating cells at 42°C for 20 min in the presence of PEG alone, PEG plus ssDNA, PEG plus LiAc, PEG plus ssDNA and LiAc, or PEG plus ssDNA and RbAc (Table 1). As shown in Table 1, we observed that ssDNA alone could enhance transformation efficiency and frequency by 7- and 6-fold, respectively; addition of LiAc increased these values by 42- and 60-fold. When used together, ssDNA and LiAc improved efficiency and frequency by 501- and 560-fold, i.e., they had a synergistic effect on both efficiency and frequency. ssDNA and RbAc in combination also displayed a synergistic effect (enhancement by 276- and 321-fold; Table 1).

Visualization of the synergistic effect of ssDNA and LiAc in combination

To clarify the mechanism underlying the synergistic effect of ssDNA and LiAc, cells were subjected to same transformation procedure used to generate Table 1, in the presence of 400 pmol of negatively charged Nanogold instead of plasmid DNA (pRS415). Negatively charged Nanogold consists of gold particles that carry a negative charge; it has been previously used as a mimic for plasmid DNA in studies of transformation (Pham, et al. 2011). Immediately after incubation at 42°C for 20 min, cells were fixed and observed using TEM. The signal from the Nanogold was amplified using GoldEnhance.

Cells incubated with PEG alone exhibited cell walls with a smooth surface (Fig. 1A), as did cells that did not receive any treatment (data not shown). In contrast, cells incubated with PEG and either ssDNA or LiAc had cell walls with slight protrusions (Fig. 1B,C). Notably, incubation of the cells with PEG, ssDNA and either LiAc or RbAc (conditions that gave the highest efficiency; Table 1), caused the cell wall structures to be the most extremely protruded, loose, and porous (Fig. 2A,B). In addition, the porous cell wall appeared to be partially removed from plasma membrane (Fig. 2A,B). Thus, taking these data together with the absence of an effect of ssDNA and LiAc on the transformation efficiency of spheroplasts (Schiestl and Gietz 1989), we concluded that the target of both ssDNA and LiAc/RbAc is the cell wall, and that the function of these compounds is to cause the structure of the cell wall to become protruded, loose, and porous (Fig. 2A,B).

The modification of the cell wall by LiAc alone (Fig. 1C) was consistent with previous reports demonstrating that LiAc alone can modify the structure of the cell wall and increase permeability to nucleic acid or YOYO-1 (Brzobohaty and Kovac 1986,

Chen, et al. 2008, Zheng, et al. 2005). Previously, we used TEM to observe intact *S. cerevisiae* cells incubated with Nanogold and PEG in the absence of LiAc and ssDNA (Pham, et al. 2011). In this study, we used TEM to visualize for the first time the synergistic effect caused by both ssDNA and LiAc/RbAc.

Negatively charged Nanogold was observed as a dot-like signal within the cell wall; Nanogold in the intracellular space was always associated with a membrane structure, i.e. Nanogold was found at the periphery of the structures within the yeast cell (Figs. 1, 2), as observed previously (Pham, et al. 2011). These observations support the idea that Nanogold enters cells via membrane invagination (Kawai, et al. 2004). It should be noted that the Nanogold appeared to be partially trapped in the pores formed in the cell wall in response to ssDNA and LiAc/RbAc (Fig. 2A,B, arrows). This led us speculate that such a binding mode, in which plasmid DNA is similarly trapped in cell wall pores, is at least one of the factors explaining the synergistic effect of ssDNA and LiAc/RbAc on transformation efficiency and frequency.

Behavior of plasmid DNA and ssDNA

To determine how plasmid DNA and ssDNA behave during transformation, we used YOYO-1. YOYO-1 is a widely used cell-impermeable fluorescent DNA probe (Gurrieri, et al. 1997); intercalation of YOYO-1 into DNA increases the probe's fluorescence intensity by more than 1,000-fold (Rye, et al. 1992). YOYO-1 has been successfully used to observe the behavior of plasmid DNA during transformation (Chen, et al. 2008, Pham, et al. 2011, Zheng, et al. 2005). However, no attempt has been made to observe the behavior of ssDNA using YOYO-1.

We incubated cells with PEG, ssDNA, LiAc, and YOYO-1 in the absence of plasmid DNA at 42°C for 20 min. The whole cell surface was stained with YOYO-1 (Fig. 3A). When the cells were incubated with PEG, LiAc, and YOYO-1 in the absence of any DNA, no fluorescent signal was observed (data not shown). Collectively, the fluorescent signal on the whole cell surface (Fig. 3A) indicated that ssDNA bound to the whole cell wall (Fig. 3A). Such binding is compatible with a previous report that detected cell-associated DNA using the diphenylamine assay (Gietz, et al. 1995). Moreover, when cells were incubated with PEG, ssDNA, and YOYO-1-labelled plasmid DNA, the whole cell surface was still stained by YOYO-1 irrespective of the presence or absence of LiAc (Fig. 3B,C) as in Fig. 3A. Thus, fluorescent microscopic observation using YOYO-1 could neither discriminate plasmid DNA from ssDNA nor visualize the synergistic effect of ssDNA and LiAc. This emphasizes the advantages of TEM analysis in elucidating the mechanism of transformation.

Negatively charged Nanogold was observed in the cell wall irrespective of the presence or absence of ssDNA (Figs. 1, 2), suggesting that even when it is bound to the whole cell wall (Fig. 3A), ssDNA would not inhibit plasmid DNA from binding to the cell wall. The binding of plasmid DNA to the cell wall is important for transformation, because it is the bound plasmid DNA, but not plasmid DNA in solution, that is proposed to enter the cells during transformation (Pham, et al. 2011). Under conditions that caused higher amounts of the plasmid DNA to be absorbed on the cell wall, transformation efficiency and frequency were both higher (Pham, et al. 2011). However, it had been previously proposed that plasmid DNA in solution enters the cell during transformation (Gietz, et al. 1995, Gietz and Woods 2001). Based on this proposal, it has also been supposed that the role of ssDNA or double-stranded carrier DNA is to

saturate the DNA-binding sites on the cell wall and to increase the probability of uptake of plasmid DNA from solution (Gietz, et al. 1995, Gietz and Woods 2001). Contrary to these hypotheses, our previous results (Pham, et al. 2011), taken together with our results obtained from TEM analysis (Figs. 1, 2) and microscopic observation (Fig. 3), clearly indicate that although ssDNA binds the cell surface, ssDNA and LiAc serve primarily to modify the structure of the cell wall itself. This is in contrast to a mechanism in which ssDNA and LiAc serve to saturate the DNA-binding sites of cell wall in order to increase the probability of uptake of plasmid DNA from solution. Thus, this study clearly demonstrates a new role for ssDNA, based on our previous reports (Pham, et al. 2011).

Nanogold was found in cell wall structures, but not on the surface of the cell wall (Figs. 1, 2), suggesting that both plasmid DNA and ssDNA can at least partially enter into the cell wall, and that ssDNA causes the cell wall to become protruded, loose, and porous. Moreover, TEM analysis conducted after removal of PEG detected Nanogold in the cells, but not on the surface of the cell wall, even in the absence of LiAc and ssDNA (Fig. 1A). This would be in good agreement with previous reports by Bruschi et al. (Bruschi, et al. 1987). In that study, the authors transformed intact *S. cerevisiae* cells by incubating the cells with plasmid DNA in the presence of PEG alone, and demonstrated that plasmid DNA was DNase-resistant after PEG was removed, but DNase-sensitive in the presence of PEG (Bruschi, et al. 1987). We ascribe the DNase-resistance of plasmid DNA to the probable location of the DNA within the structures of the cell wall, as observed for Nanogold (Fig. 1).

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Figure legends

Fig. 1. Visualizing the effects of ssDNA and LiAc. Cells were incubated at 42°C for 20 min in the presence of negatively charged Nanogold, with PEG (A), PEG plus ssDNA (B), or PEG plus LiAc (C), treated, and observed by TEM. Left panel indicates the image at 12,500-fold magnification; scale bar is 2 μm . A portion of the left panel image is further magnified and shown in the right panel (47,800-fold magnification; scale bar is 0.50 μm). Signals from Nanogold were enhanced using GoldEnhance, and are observed as dots.

Fig. 2. Visualization of the synergistic effect of ssDNA and either LiAc or RbAc. Cells were incubated at 42°C for 20 min in the presence of negatively charged Nanogold, with PEG plus ssDNA and LiAc (A) or PEG plus ssDNA and RbAc (B), treated, and observed by TEM. Images are displayed as in Fig. 1. The negatively charged Nanogold appeared to be partially trapped in spaces formed by porous structures (arrows) in the cell wall that formed in response to ssDNA and LiAc/RbAc.

Fig. 3. Behavior of ssDNA visualized using YOYO-1. Cells were incubated at 42°C for 20 min in the presence of PEG and ssDNA with (A) LiAc and YOYO-1 alone, (B) LiAc and YOYO-1-labelled plasmid DNA, or (C) YOYO-1-labelled plasmid DNA alone. After incubation, cells were gently washed in sterilized water and observed by fluorescence microscopy.

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Table 1. ssDNA and LiAc in combination synergistically enhance transformation efficiency and frequency

Composition ^a	Transformation efficiency		Viable cells		Transformation efficiency (A/B)
	cfu/μg pRS415 (A)	fold	cfu (x 10 ⁴) (B)	fold	fold
None ^b	2,008 ± 1,606	1	1,592 ± 8	1.00	1
ssDNA ^c	13,613 ± 8,226	7	1,821 ± 141	1.14	6
LiAc ^d	84,888 ± 37,692	42	1,128 ± 400	0.71	60
LiAc + ssDNA ^e	1,007,500 ± 657,319	501	1,428 ± 76	0.90	560
RbAc + ssDNA ^f	555,625 ± 239,194	276	1,372 ± 220	0.86	321

^a The cells were incubated together with 0.2 μg pRS415 at 42°C for 20 min in 42 μl suspension containing 36% PEG alone ^b, 36% PEG plus 0.29 mg/ml ssDNA ^c, 36% PEG plus 10.7 mM LiAc ^d, 36% PEG plus 10.7 mM LiAc, and 0.29 mg/ml ssDNA ^e, 36% PEG plus 10.7 mM RbAc, and 0.29 mg/ml ssDNA ^f

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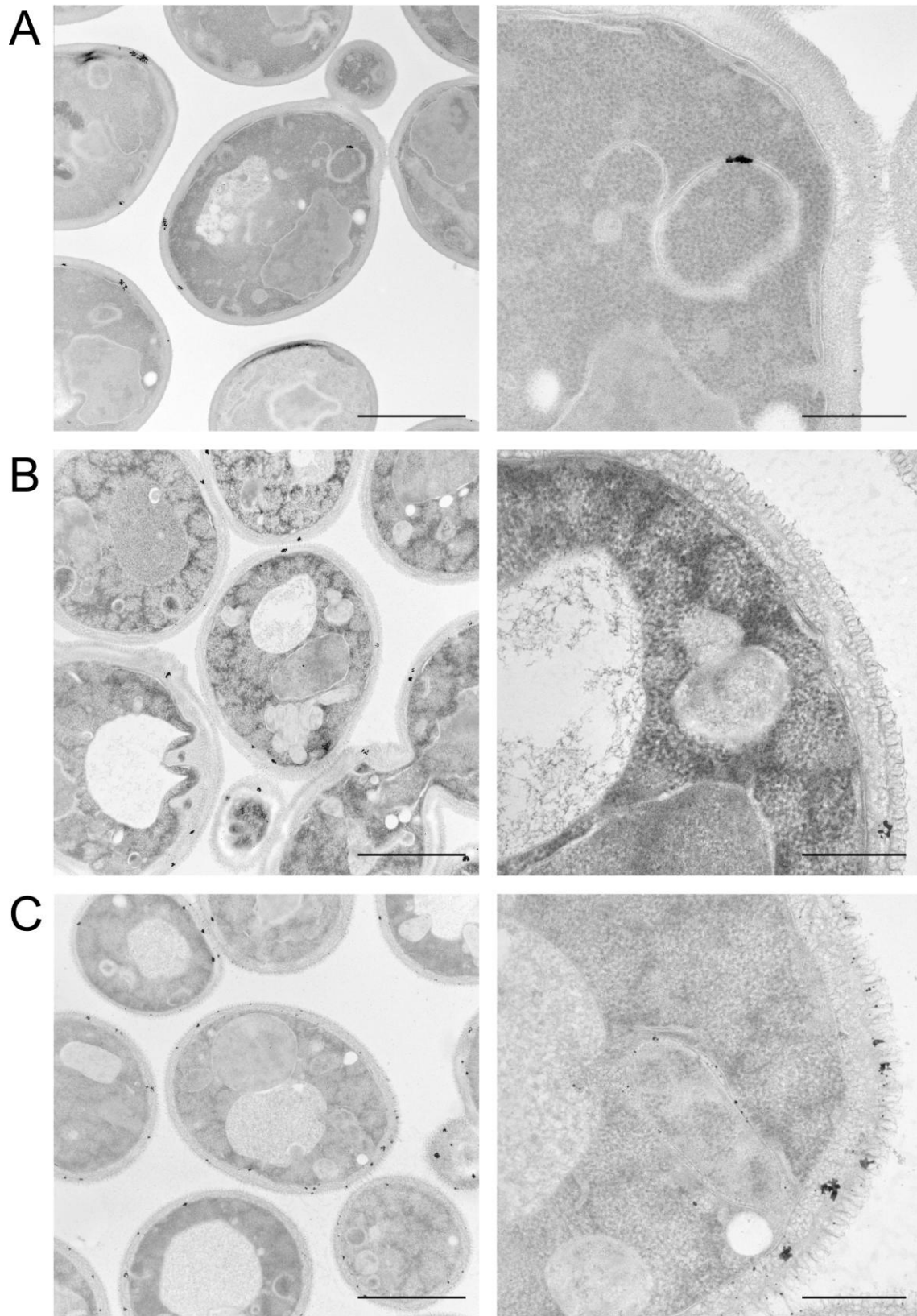


Fig. 1 Tuan et al.

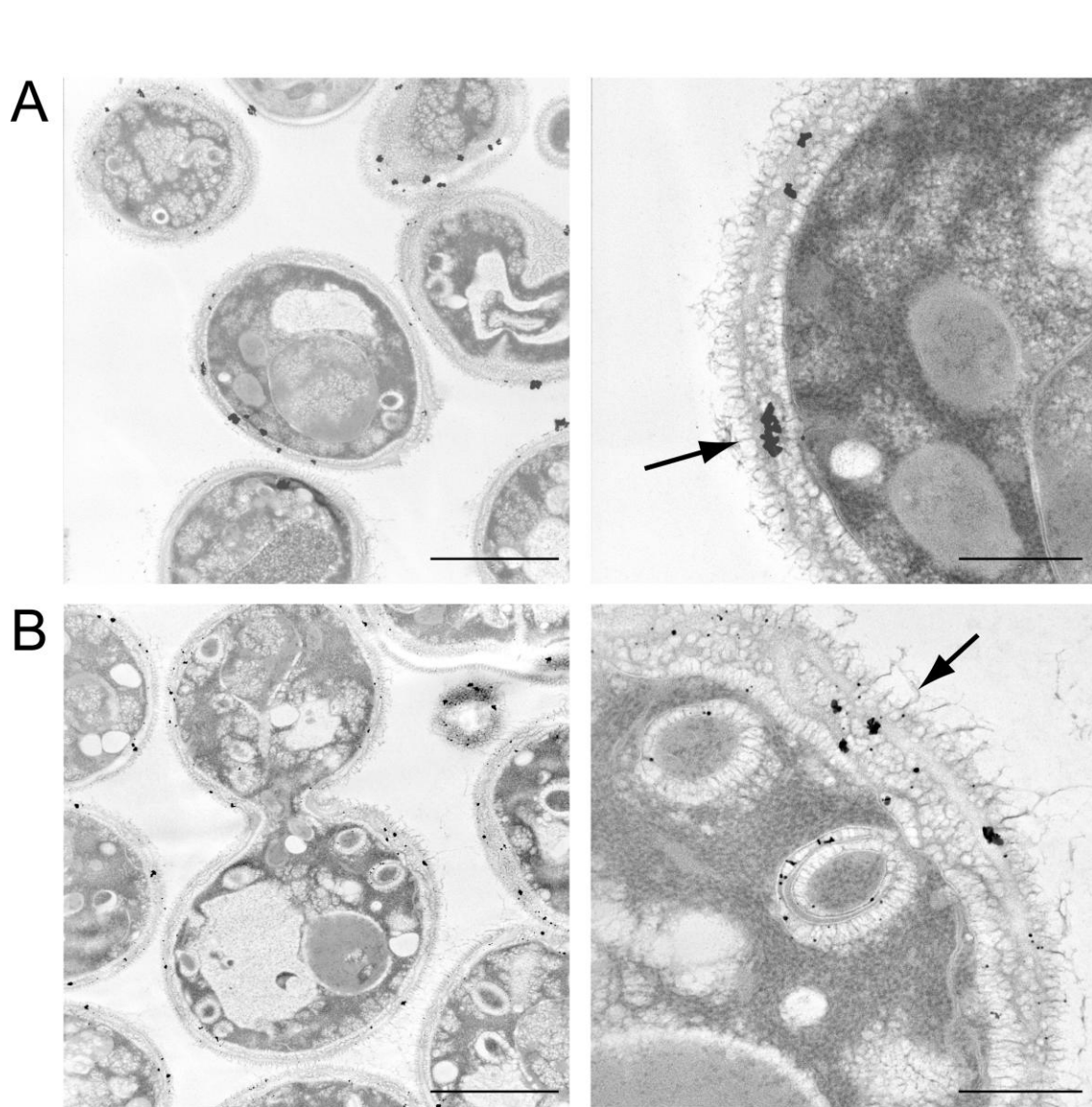


Fig. 2 Tuan et al.

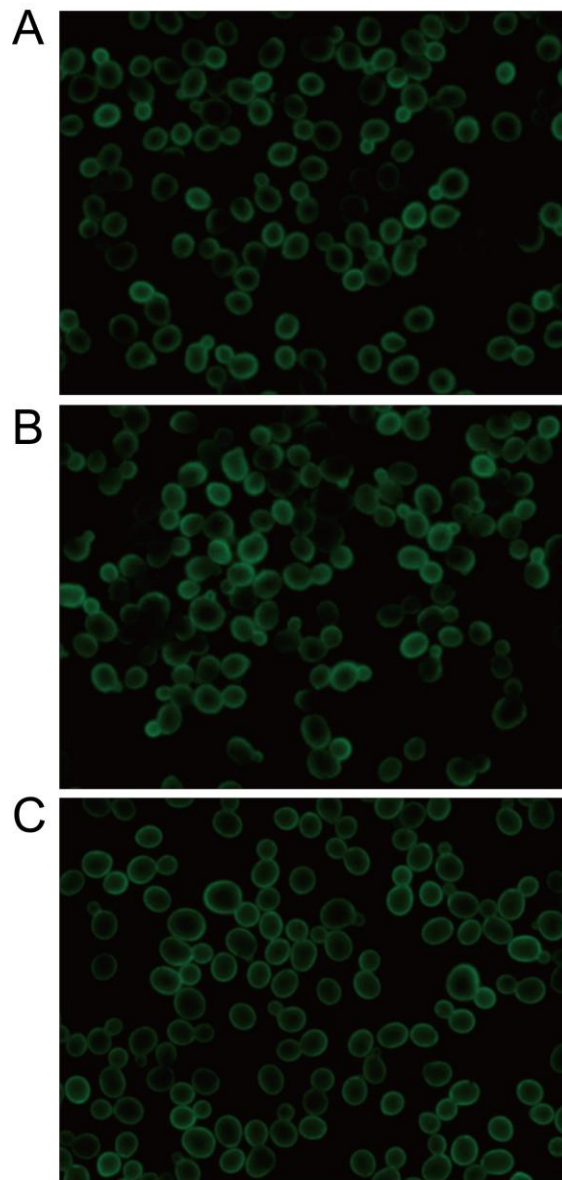


Fig. 3 Tuan et al.

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